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Biocorrosion of Copper Metal by *Aspergillus niger*

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ABSTRACT

Several geoactive fungi were investigated for their biocorrosion impact on metallic copper, to further understanding of the potential roles that fungi may have in the biotransformation of such substrate, and the mechanisms involved. Copper metal showed little toxicity and test fungi were able to grow in direct or indirect contact with copper and to colonize copper sheet. *A. niger* was able to biodeteriorate copper metal through proton- and ligand-mediated dissolution mechanisms, leading to significant mass loss and surface etching. The formation of a secondary copper oxalate (moolooite) biomineral crust together with cuprite deposition lead to alteration of surface topography and visual appearance, highlighting the significance of oxalate excretion in effecting fungal metal biotransformations. The metal transforming influence of fungal colonization may have some implications for biodeterioration, protection and preservation of cultural relics and artefacts as well as certain components of the built environment.

Keywords: *Aspergillus niger*; copper; biocorrosion; oxalate; biomineralization

1. Introduction

Metal loss and damage in the built environment resulting from corrosion is one of the major causes of structural deterioration, economic loss and cultural damage (Crispim and Gaylarde, 2005). It is estimated that damage caused by all forms of corrosion costs about 2 trillion pounds (UK £ sterling) annually, which is about 3% of the global gross domestic product (Bhandari et al., 2015). Microbial colonization can have a significant influence on the built environment, showing all kinds of effects such as discolouration and staining, biocorrosion, and biodeterioration of metallic, organic and inorganic components (Warscheid and Braams, 2000; Scheerer et al., 2009; Sterflinger and Piñar, 2013; Gadd, 2017b). This can pose serious concerns for built infrastructure, including nuclear waste storage facilities, oil storage tanks and sewer systems, as well as human habitation and cultural artefacts (Nica et al., 2000; Gu, 2007; Herrera and Videla, 2009; Turick and Berry, 2016).

The growth of diverse organisms, including bacteria, lichens, and fungi, can have significant effects on structural materials derived from rocks, minerals and metals (Gadd, 2007; 2017a,b) Several studies have concentrated on the involvement of bacteria, including sulfate-reducing, iron-reducing, sulfur-oxidizing, and iron-oxidizing genera, in metal biocorrosion and concrete biodeterioration (Emde et al., 1992). However, relatively little attention has been paid to fungi despite them often being the most visible and destructive of microbiota in the built environment because of their biodeterioration of a wide variety of substrates, including wood, plastics and rock and mineral-based building components (Sterflinger, 2010; Onofri et al., 2014; Gadd, 2017a) Many fungal species are capable of solubilizing metals from metal-bearing minerals and substrates (Gadd, 1993, 2017a,b; Fomina et al., 2005a,b; 2007). Some early work showed that

53 *Penicillium spp*. were able to solubilize and accumulate zinc, aluminum, copper, and lead (Siegel
54 et al., 1983), while *Cladosporium resinae* was involved in the biodeterioration of aluminium
55 (Iverson, 1987). The relative lack of information on copper biocorrosion by fungi contrasts with
56 the extensive literature on responses of wood decay fungi to copper in connection with the
57 application of copper compounds as wood preservatives (Schilling and Jellison, 2006; Freeman
58 and McIntyre, 2008; Zelinka et al., 2019a,b). Many such fungi exhibit marked tolerance to copper
59 with the formation of copper oxalate acting as a detoxification process and underpinning copper
60 tolerance (Clausen and Green, 2003; Green and Clausen, 2003; Hastrup et al., 2005; Kartal et al.,
61 2015; Ohno et al., 2015; Karunasekera et al., 2019).

62 Copper and its alloys have important uses in interior and exterior environments (Elwell and
63 Scholes, 1967) and has been used for centuries in architectural and cultural applications, e.g.
64 statues, ornaments and buildings (Frankfort, 1956; de la Fuente et al., 2008). Furthermore,
65 because of good machinability and conductivity, copper is extensively used in the electronics,
66 communications and digital industries, such as in circuit boards, connections and terminals, and
67 also widely utilized in heat exchangers or conductors. To date, most attention has focussed on
68 atmospheric abiotic corrosion of copper and although fungal bioweathering of copper-containing
69 minerals has been studied (Fomina et al., 2005a,b, 2007, 2017) biocorrosion of copper metal by
70 fungi has received little attention. The objective of this research was therefore to investigate the
71 ability of fungi to mediate biocorrosion of copper metal. In this work, several fungi with known
72 metal and mineral transformation abilities, i.e. *Aspergillus niger* (Sayer and Gadd, 1997; Horeh et
73 al., 2016; Fomina et al., 2017; Ferrier et al., 2019; Kang et al., 2019, 2020; Suyamud et al., 2020),
74 *Beauveria caledonica* (Fomina et al., 2005a) and *Paecilomyces javanicus* (Rhee et al., 2012, 2016),

were used to investigate interactions with copper metal to gain understanding of the roles that fungi may play in the biocorrosion or biotransformation of such a material, effects on the copper substrate and the mechanisms involved.

2. Materials and Methods

2.1 Organisms and media

Aspergillus niger (ATCC 1015), *Beauveria caledonica* (provided by G, Genney (CEH Merlewood collection)) and *Paecilomyces javanicus* (Friedrichs & Bally; A.H.S. Brown & G. Smith) were maintained on malt extract agar (MEA) plates, (Merck, Darmstadt, Germany) at 25°C in the dark. *A. niger* was grown for 3 - 4 days prior to experimental subculture: *B. caledonica* and *P. javanicus* were grown for at least one week prior to experimentation.

AP1 media (in Milli-Q water) comprised 38 mM $(NH_4)_2SO_4$ (Alfa Aesar, Ward Hill, USA) or 59 mM $NaNO_3$ (Acros, New Jersey, USA), 3.7 mM KH_2PO_4 (Acros, New Jersey, USA), 0.8 mM $MgSO_4 \cdot 7H_2O$ (BDH, Poole, UK), 0.2 mM $CaCl_2 \cdot 6H_2O$ (BDH, Poole, UK), 1.7 mM NaCl (Sigma-Aldrich, St. Louis, USA), 9×10^{-3} mM $FeCl_3 \cdot 6H_2O$ (Sigma-Aldrich, St. Louis, USA), and trace metals 1.4×10^{-2} mM $ZnSO_4 \cdot 7H_2O$ (BDH, Poole, UK), 1.8×10^{-2} mM $MnSO_4 \cdot 4H_2O$ (Sigma-Aldrich, St. Louis, USA), and 1.6×10^{-3} mM $CuSO_4 \cdot 5H_2O$ (BDH, Poole, UK), 111 mM D-glucose (VWR, Lutterworth, UK). Modified Czapek-Dox agar (MCD) media was prepared of the following composition: 166 mM D-glucose (VWR, Lutterworth, UK), 35 mM $NaNO_3$ (Acros, New Jersey, USA), 7 mM Na_2HPO_4 (Acros, New Jersey, USA), 2.0 mM $MgSO_4 \cdot 7H_2O$ (BDH, Poole, UK), 7 mM KCl (BDH, Poole, UK), 0.04 mM $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich, St. Louis, USA), 15 g L^{-1} agar No.1 (Oxoid, Basingstoke, UK). All stock solutions were sterilized separately by autoclaving at 121 °C for 15 min and subsequently mixed with sterile (115 °C, 15 min) D-glucose solution. The media were adjusted to pH 5.5 using 1 M HCl before sterilization by autoclaving at 115 °C for 15 min. For solid media, 15 g L^{-1} agar No.1 (Oxoid, Basingstoke, UK) was used. Liquid media was inoculated using a spore suspension in sterile Milli-

99 Q water, taken from a freshly grown MEA slope, to an initial concentration of 5×10^5 spores mL^{-1}
100 (ME) and 1×10^6 mL^{-1} (AP1). Flasks were incubated in a shaking incubator (Infors Multitron II,
101 Infors HT, Bottmingen, Switzerland) at 125 rpm in the dark at 25 ^oC.

102 Waste computer power cables were used as a copper source for the experiments. After removal
103 of plastic coatings, the bare copper wire was cut into 2-3 mm long pieces which were oven-
104 sterilized at 105^oC for 48 h. 10 cm of copper wire weighed ~800 mg: the purity of the copper was
105 not evaluated. Copper wire pieces were distributed over the agar surfaces either between the
106 agar and a cellophane membrane placed on top, or on a cellophane membrane (Louth, Focus
107 Packaging and Design Ltd, Louth, UK; thickness 27.5 µm) placed on top of the agar (Sayer & Gadd,
108 1997). The membranes allow the transfer of diffusible nutrients or metabolites between the agar
109 and the fungus, provide a means of easily removing the biomass, and therefore providing
110 information on direct and indirect interactions between the organism and substrate (Sayer and
111 Gadd, 1997; Suyamud et al., 2020). Distribution densities were ~15-20 pieces cm^{-2} on MEA but,
112 because of enhanced toxicity, 8 copper wire pieces were evenly distributed on AP1 or MCD agar
113 plates. For copper metal sheet colonization experiments, copper sheet pieces (~ 0.5 × 0.5 x 0.07
114 cm) (R.I.C.E. Metals Ltd, Truro, UK) were abraded with 100-grit aluminium oxide abrasive paper
115 to enhance fungal colonization, washed with 1% nitric acid and subsequently sterile Milli-Q water.
116 Four pieces of scratched copper sheet were placed above the cellophane membrane on agar
117 media in 9-cm diameter Petri dishes. Inoculation of test fungi, at the centre of the agar plates,
118 was achieved by using 6 mm diameter discs of mycelium cut from the periphery of growing
119 colonies on MEA plates (Sayer & Gadd, 1997). Four copper sheet pieces were distributed

120 symmetrically on the agar surface, and plates (at least three replicates) were incubated in the
121 dark at 25 ^{o}C .

122

123 ***2.2 Metal tolerance and pH determination***

124 Measurements were taken of colony diameter at regular time intervals in order to assess growth
125 rates and possible inhibitory effects (Gadd et al., 1985; Sayer et al., 1995). Biomass was removed
126 from the membrane overlying the agar surface using a scalpel and dried to constant weight at
127 105 ^{o}C for at least 4 days. Metal tolerance was evaluated using a tolerance index (TI) based on
128 the dry weight of fungal biomass as follows: TI = (dry weight of treated mycelium/ dry weight of
129 the control mycelium) ×100% (Sayer et al., 1995). The pH of the agar surface, after the fungal
130 colony had covered the surface completely, was measured at 6 equidistant intervals across plate
131 axes using a flat probe pH electrode (VWR International, Lutterworth, UK) after removal of the
132 cellulose membrane and biomass.

133

134 ***2.3 Organic acid measurements***

135 *A. niger* was incubated in malt extract (ME) or AP1 liquid media amended respectively with 1%
136 and 0.05% (w/v) copper wire pieces at 25^{o}C in the dark on an Infors II Multitron shaking incubator
137 (125rpm). 1 mL aliquots of supernatant were collected at 0, 7, 14, and 21 days after fungal
138 inoculation and filtered through a 0.2 µm pore diameter cellulose acetate membrane filters
139 (Whatman, Maidstone, UK). The acids were analysed using a BioRad Aminex HPX-87X-87H ion

exclusion column (300 mm × 7.8 mm) fitted with a Micro-Guard Cation H Refill guard column (BioRad, Richmond CA, USA) at 35 ^{o}C on a DIONEX UltiMate 3000 system (ThermoFischer Scientifics, Germering, Germany) including a pump, degasser, autosampler, and variable wavelength detector. The sample injection volume was 20 µL and flow rate (5 mM H_2SO_4) was 0.6 mL/min. Detection was carried out at 210 nm for 18 min. Acids were identified and quantified by their specific retention times and peak areas of the following standards: oxalic, citric, fumaric, gluconic, itaconic, malic and succinic acid.

2.4 Elemental and mineralogical analysis

The pieces of copper wire were separated from the agar after removal of fungal biomass by homogenizing the agar in Milli-Q water at 80^{o}C, and repeating washing of the wire pieces in warm Milli-Q water. The copper samples were dried in a desiccator at ambient temperature for at least 3 weeks prior to analysis. Copper sheet was cleaned by gently washing with 1% Triton (v/v_{aq}) and water for 24 h in 50 mL centrifuge tubes on a SB Tube Rotator (20 rpm), and kept in a desiccator prior to further examination. Mineralogical and elemental analyses were carried out using energy dispersive X-ray analysis (EDXA) coupled with scanning electron microscopy (SEM) and X-ray diffraction (XRD) (see Li & Gadd, 2017; Ferrier et al., 2019; Suyamud et al., 2020; Kang et al., 2020; Yang et al., 2020).

2.5 Statistical analysis

Origin 9.1 was used, and at least three replicate determinations were used in experiments.

3. Results

3.1 Effect of copper metal on fungal growth

A. niger, B. caledonica, and P. javanicus were all able to grow on copper metal amended media. *A. niger* grew the fastest, while the growth of *B. caledonica* and *P. javanicus* was much slower. The inclusion of copper metal in the medium had little significant effect on growth rates of the test fungi (Table 1). The presence of copper metal slightly inhibited colony expansion of *A. niger* on AP1 medium, while growth of *B. caledonica* was slower on copper metal-amended MEA and MCD medium than on the corresponding controls. While *P. javanicus* grew slowly, the presence of copper metal had little effect. The type of contact of the test fungi with the copper metal showed different effects on growth. The growth rate of *A. niger* when directly interacting with copper metal on the cellophane membrane surface on MEA and ammonium salt AP1 medium appeared to be slightly inhibited, compared with the treatments where copper metal was incorporated in the agar below the cellophane membrane, perhaps due to higher exposure to mobile copper species in proximity to the metal. Similar effects occurred with *B. caledonica* on MCD and *P. javanicus* on nitrate salt AP1 medium (Table 1). Growth in the absence or presence of copper wire metal pieces was also expressed as a tolerance index (TI) based on the yields of fungal biomass, which confirmed that the effects of copper metal varied among the fungal species. On MEA, the TIs for *A. niger* were all around 100% in the presence of copper metal both when below and above the cellophane membrane. The growth of *P. javanicus* on nitrate salt AP1 and MCD medium containing copper metal above or below membrane showed similar significant reductions (TI =70.3%-75.9%, Table 2). Compared with the TI values for *B. caledonica* on nitrate salt AP1 medium (around 90%), the TI values for MCD revealed a remarkable reduction to about

60%. The contact mode with the copper showed little effect on the TI values except for *B. caledonica* on MEA media: the TI for indirect contact was reduced by 16.5% compared to direct contact. This showed that copper metal had a significant influence on growth of *B. caledonica*, especially when in direct contact with the metal.

3.2 pH changes in media after fungal growth

The medium pH decreased during growth of *A. niger*, compared with abiotic controls, while the pH increased slightly in the presence of copper metal compared to the negative control (Table 3). This showed that some acids produced by the test fungus were consumed by the copper metal resulting in an increase in pH. Of the different media inoculated with *A. niger*, the pH of ammonium salt AP1 medium reached the lowest value. For *B. caledonica* and *P. javanicus*, the medium become alkaline during growth, and the pH markedly increased in the presence of copper metal compared with the abiotic controls (Table 3).

3.3 Determination of organic acids

For ME liquid media, oxalic, malic and succinic acid were the main organic acids detected: citric, fumaric, gluconic and itaconic acid were not detected. Oxalic acid dominated, the highest amount (12.3 mM) appearing after *A. niger* was incubated with copper for 14 days. In the absence of copper metal, corresponding oxalic acid production was 7.8 mM (Fig.1a) indicating the presence of copper metal stimulated the production of oxalate. After 14 days, the concentration

of oxalic acid decreased, probably the result of oxalate consumption by the formation of copper oxalate (see later). The secretion of malic acid reached a peak after 7 days when *A. niger* was grown in ME liquid media and then remained constant. The presence of 1% (w/v) copper stimulated the generation of malic acid up to 2.2 mM (Fig.1b). Succinic acid occurred at similar concentrations both in the control and 1% copper metal treatment and decreased over incubation time (Fig. 1c). For copper-containing ammonium AP1 liquid media, only limited malic acid was detected during incubation of *A. niger* with 0.05% (w/v) copper metal, less than in the control, and increased gradually reaching equilibrium at 0.025 mM (Fig. 1d). The contents of other organic acids, including oxalic acid, were below the detection limits.

3.4 Biocorrosion and biomineral formation

The capacity of *A. niger* to solubilize copper metal was manifest by the reduced diameter of copper wire pieces in the ammonium salt AP1 medium. To examine whether there was any medium influence on the copper metal, control copper wire pieces were collected from agar plates containing the same amount of copper metal incubated in the absence of fungi. In this comparison, the solubilization of copper metal by *A. niger* was clearly observed (Fig. 2). The diameter of control copper wire pieces was 189.6 ± 0.4 μm, while the diameter of *A. niger* treated copper wire was 75.8 ± 1.6 μm, a significant decrease of 60.0%. Such a decrease in the diameter of the wire as a result of fungal action clearly reflects significant loss of mass. In addition, the surface appearance of fungal exposed copper metal was rougher than the control.

Compared with abiotic copper sheet samples, significant alteration of the copper sheet surfaces was observed and a blue crust was evident on visual examination (result not shown). Microscopic examination showed that distinct biocorrosion patterns were observed on colonized copper sheets, incubation with *A. niger* resulted in etching and disruption over the surface (Fig. 3a). Some of the etched channels showed a similar pattern to fungal mycelium grown on the surface which mirrored colonization and branching by the fungal hyphae. The dimensions of etching traces varied, their width being around 1 - 3.5 µm, while the width of fungal hyphae growing on a copper sheet surface was ~10 µm (Fig.3b).

For fungi grown in the presence of copper wire pieces, it was found that there was obvious evidence of biomineral formation on the copper surfaces and different biocorrosion patterns produced by *A. niger*. The varied contact modes resulted in different shapes of the secondary minerals produced probably reflecting differences in the secretion of geoactive metabolites. Microscopic examination showed widespread corrosion of the copper wire pieces as well as the formation of various crystalline structures. Different patterns of biominerals were produced by *A. niger*. The secondary minerals formed on copper pieces incorporated in agar below the membrane showed a lamellar structure (Fig. 4a, b). The size of these biominerals was approximately 3-4 µm in diameter. The biominerals that formed on copper wire pieces which were in direct interact with *A. niger* showed some different morphologies, the most distinctive being layered structures similar to the crystals formed on the copper wire pieces that had indirect contact with *A. niger*. Some other structures showed flakiness, and some were amorphous. Apart from these, some mycelial encrustations were observed on the surface of copper wire pieces (Fig. 4c, d). EDXA showed that the biominerals formed on copper pieces contained copper, carbon,

246 and oxygen (Fig. 5a, c). As shown in Fig. 6, X-ray diffraction analyses of the biominerals produced
247 by *A. niger* confirmed the presence of moolooite ($CuC_2O_4 \cdot 0.4H_2O$) (ICPDS Card NO. 21 - 297) and
248 cuprite (Cu_2O) (ICPDS Card NO. 05 - 667).

249

4. Discussion

Fungal colonization can have an extraordinary biodeteriorative influence on organic as well as metallic and mineral constituents of the built infrastructure, and historical artefacts, such as staining, biocorrosion, and biodeterioration (Miller et al., 2012; Gadd, 2017a; Gadd and Dyer, 2017). Although the tolerance and responses of fungi to soluble copper, especially for wood decay fungi, has been widely reported, and investigations carried out on the corrosion of metals embedded in copper preservative-treated wood, such as aluminium and steel, (Zelinka and Rammer, 2009; Zelinka and Stone, 2011), or copper fasteners in wood (Zelinka et al., 2019a,b), the influence of fungi on metallic copper biocorrosion has received little attention. Copper is an essential element for fungal growth. It can act as an enzyme cofactor and is essential for respiration, free radical detoxification, and iron acquisition (Antsotegi-Uskola et al., 2020). However, excess copper accumulation will result in toxicity, hence its historical and current use as a fungicide and wood preservative in inorganic and organic forms (Freeman and McIntyre, 2008; Lamichhane et al., 2018). Copper ions can inactivate metalloenzymes by metal displacement, bind to intracellular sulfur, oxygen, and nitrogen ligands, and also promote the production of reactive oxygen species (ROS) (Fridovich, 1983; Macomber and Imlay, 2009; Smith et al., 2017). However, many fungal species can show pronounced tolerance to copper, particularly under acidic conditions (Gadd and Griffiths, 1980a; Gadd and White, 1985; Green and Clausen, 2005; Humar et al., 2005; Ohno et al., 2015) or when in insoluble forms (Karamushka et al., 1996; Fomina et al., 2017), and the copper-tolerance of *A. niger* and *B. caledonica* have been reported previously (Gharieb et al., 2004; Fomina et al., 2005a; Iskandar et al., 2011). In the built environment, including cultural heritage, insoluble copper-containing substrates include metallic

copper and alloys, copper-containing minerals, preservatives and pigments. Biodeteriorative effects therefore depend on direct and indirect interactions that result in release of Cu^{2+} which can interact with organisms, bind to environmental constituents, or form copper-containing secondary minerals with organic and inorganic ligands, all further contributing to biodeteriorative effects and alteration of appearance. In this work, the test fungi could all grow in the presence of copper metal, whether in direct or indirect contact, and although there was some variation in response, little significant toxicity was manifest. For *A. niger*, inhibition of growth rate was slightly greater with direct contact with the copper, although this was marginal and not clear for the other test fungi. This is unsurprising since the cellophane membrane would not have acted as a barrier to mobile copper species. Tolerance indices (TIs) largely reflected these results with little effect on *A. niger*, but some significant reduction for *B. caledonica* on MCD and *P. javanicus* on MCD and NO_3^--AP1 although, due to slower growth, TIs were derived from a much longer incubation time than for *A. niger*. These data show that the test fungi could grow successfully in the presence of metallic copper despite some limited toxicity.

Fungi can effect mineral solubilization through proton and ligand-mediation dissolution mechanisms (acidolysis and complexolysis, respectively) as well as redox reactions (redoxolysis) (Burgstaller and Schinner, 1993; Gadd, 2007, 2010; Gadd et al., 2014) and such mechanisms will also be involved for metallic substrates (Fomina et al., 2008; Rhee et al., 2012, 2014, 2016). It seems that acidolysis and complexolysis were the main mechanisms operating in this study. Many examples of mineral solubilization by fungi are correlated with a pH decrease (Sayer and Gadd, 1997; Fomina et al., 2004, 2005b) that can result from proton excretion, nutrient-proton antiport, ammonium utilization, organic acid secretion, and respiration (Burgstaller and Schinner, 1993)

The pH is a vital factor in mineral transformations by fungi because of its significant effects on metal biosorption and transport processes, and the nucleation and precipitation of secondary mineral products (Burford et al., 2003, 2006; Parvathi et al., 2007; Wei et al., 2012) as well as effects on fungal growth and nutrition, including organic acid excretion (Gadd, 1999; Fomina et al., 2004; Gadd, 2010). An acidic pH can also lead to a marked reduction in the toxicity of soluble copper to fungi (Starkey, 1973; Gadd and Griffiths, 1980b) due to decreased sorption and intracellular accumulation of copper at low pH (Gadd and White, 1985). In this work, the media pH significantly decreased with *A. niger*, particularly with ammonium as nitrogen source, as noted in other studies (e.g. Fomina et al., 2017). In contrast, growth of *B. caledonica* and *P. javanicus* resulted in increased alkalinity, especially when grown using nitrate as nitrogen source (Lapeyrie et al., 1987; Gadd, 1999), and influence copper speciation as hydroxides and carbonates which will tend to reduce potential copper toxicity. In acidolysis, oxygen atoms on the metal surface are protonated to water leading to Cu^{2+} release (Burgstaller and Schinner, 1993), as simplified in equation 1:

$$2Cu + 4H^+ + O_2 \rightarrow 2Cu^{2+} + 2H_2O \quad (1)$$

The secretion of low molecular weight organic acids is often pivotal to mineral and metal transformations, potentially contributing to acidolysis, complexation and redox interactions (Gadd, 1993; Fomina et al., 2005a; Gadd et al., 2014) and *A. niger* can generate several different organic acids, dependent on nutritional conditions, that are effective for metal complexation, e.g.

315 citric and oxalic acid (Gadd, 1999; Ruijter et al., 1999). The production of such acids by *A. niger*,
316 and other *Aspergillus* species, has been investigated for metal bioleaching and biorecovery from
317 electronic wastes (Kolenčík et al., 2013; Horeh et al., 2016), spent refinery catalysts (Santhiya and
318 Ting, 2005), mine tailings, deposits and ores (Mulligan et al., 2004; Seh-Bardan et al., 2012;
319 Mohanty et al., 2017; Kang et al., 2019, 2020; Yang et al., 2019, 2020) . The reduction of metal
320 species in redoxolysis can be mediated by excreted metabolites, and oxalic acid is capable of Fe(III)
321 and Mn(IV) reduction to Fe(II) and Mn(II) respectively (Dutton and Evans, 1996; Gadd, 1999; Wei
322 et al., 2012). In this work, the main organic acids secreted by *A. niger* in ME medium were oxalic,
323 malic and succinic acid with oxalic acid dominating, the presence of copper appearing to enhance
324 oxalate production. This has been observed in wood decay fungi which can show high levels of
325 oxalate production (Hastrup et al., 2012) and an important mechanism of detoxifying copper in
326 preservative-treated wood was overexcretion of oxalic acid (Clausen and Green, 2003; Green and
327 Clausen, 2005; Hastrup et al., 2012; Ohno et al., 2015). *Beauveria caledonica* also showed oxalate
328 overexcretion in the presence of toxic metal-containing minerals, including those of copper
329 (Fomina et al., 2005a).

330 In complexolysis, the metal is solubilized from the substrate due to the complexing capacity of
331 the complexant molecule, and this can also promote the solubility of a metal ion which has been
332 detached from metal via acidolysis (Burgstaller and Schinner, 1993). Oxalate will also precipitate
333 with many metal species, apart from alkali metals, and the formation of copper oxalate has often
334 been considered as a tolerance mechanism in fungi (Murphy and Levy, 1983; Dutton and Evans,
335 1996; Gadd, 1999; Clausen et al., 2000; Green and Clausen, 2003, 2005; Jarosz-Wilkołazka and

Gadd, 2003; Hastrup et al., 2005; Ohno et al., 2015). The reactions between $Cu^{2+}$and oxalic acid can be summarized as follows (Horeh et al., 2016):

$$C_2H_2O_4 \rightarrow C_2HO_4^- + H^+ \quad (2)$$

$$C_2HO_4^- \rightarrow C_2O_4^{2-} + H^+ \quad (3)$$

$$2[C_2HO_4^-] + Cu^{2+} \rightarrow Cu[C_2HO_4]_2 \quad (4)$$

$$C_2O_4^- + Cu^{2+} \rightarrow CuC_2O_4 \quad (5)$$

The amount and variety of organic acids excreted by *A. niger* is highly dependent on medium composition, especially carbon and nitrogen source, pH, and buffering capacity (Burgstaller and Schinner, 1993; Dutton and Evans, 1996; Gadd, 1999; Palmieri et al., 2019). A relationship between the nitrogen source and oxalate excretion has often been demonstrated (Dutton and Evans, 1996; Gadd, 1999; Fomina et al., 2017). In ammonium salt AP1 medium, only limited malic acid was detected in this work but the medium became strongly acidic because ammonium assimilation leads to the production of protons which reduces the pH of the extracellular environment (Sazanova et al., 2015). In addition, a low external pH restricts fungal production of oxalic acid (Roos and Luckner, 1984; Ruijter et al., 1999) since a key enzyme responsible for oxalate formation, oxaloacetate acetylhydrolase (OAH), is inhibited at low pH values (Gadd, 1999; Ruijter et al., 1999). It seems clear that acid dissolution and complexation were the main processes mediated by *A. niger* in this work, which could have dramatic effects on metallic copper

as evidenced by the marked solubilization of copper wire and the etching and disruption of copper sheet surfaces.

Besides the dissolution of metal-bearing substrates, fungal biodeterioration can also occur through secondary mineral formation, and oxalates are frequently associated with the disruption and flaking of outer layers of building components, plaster, frescoes etc. (Fomina et al., 2010; Gadd, 2017b; Gadd et al., 2014). Conversely, oxalate formation in other contexts can stabilize external surfaces through involvement in stable patina formation emphasising that biodeteriorative or surface effects are highly dependent on the substrate and physico-chemical conditions (Gadd, 2017a, b; Gadd et al., 2014; Palmieri et al., 2019). In this work, a vivid blue crust resulted on the surface of copper metal after growth of the selected fungi, and this was also identified as the hydrated copper oxalate, moolooite, together with cuprite. Clearly, such interactions will change the appearance of the copper metal substrate and remove any metallic lustre. Copper oxalates are frequently identified on surfaces of outdoor bronze structures (Graedel et al., 1987) forming insoluble stable patinas even in an acidic atmosphere (Marabelli and Mazzeo, 1993). Innovative research has therefore explored the application of oxalate formation for protection and conservation of historic and contemporary metal artefacts, with copper oxalate appearing particularly applicable for such a purpose (Joseph et al., 2012a, b).

Several fungal species have previously been shown to be capable of copper-containing mineral transformations, and copper oxalate is frequently associated with fungal interactions with both soluble and insoluble copper-containing compounds and substrates (Murphy and Levy, 1983; Dutton and Evans, 1996; Gadd, 1999; Clausen et al., 2000; Green and Clausen, 2003, 2005; Jarosz-Wilkołazka and Gadd, 2003; Fomina et al., 2005a, 2017). It has also been reported that copper

salts can stimulate oxalate production (Green and Clausen, 2003). Extracellular copper oxalate (moolooite, $CuC_2O_4 \cdot nH_2O$ (n~0.4-0.7)) precipitation occurred on *Beauveria caledonica* hyphae and cords growing with copper phosphate (Fomina et al., 2005a, 2010).

5. Conclusions

In summary, this work has demonstrated that *A. niger* is capable of colonization and biodeterioration of metallic copper through dissolution activities and the formation of secondary copper oxalate biominerals leading to alteration of surface topography and visual appearance. Dissolution by *A. niger* can lead to significant loss in mass as evidenced by the dramatic size reduction in copper wire exposed to fungal metabolite excretion and etching of sheet copper. Little toxicity was manifest to the test organisms, and acidolysis and complexation were the significant biodeteriorative mechanisms that lead to copper oxalate crust formation. The findings emphasize the importance of oxalate excretion in effecting metal and mineral transformations, as shown in several relevant studies (Fomina et al., 2008; Gadd et al., 2014; Ferrier et al., 2019; Kang et al., 2019, 2020; Suyamud et al., 2020). It is clear that the transforming influence of fungal colonization may have some implications for biodeterioration, protection and preservation of cultural relics and artefacts as well as certain components of the built environment.

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Conflict of Interest Disclosure

The authors declare no competing financial or non-financial conflicts of interest.

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640

641 Table 1. Growth rates (mm day^{-1}) of test fungi on copper metal amended malt extract agar
642 (MEA), AP1 and MCD media. Results are shown as the growth rates on copper metal amended
643 or unamended medium calculated by linear regression. Test fungi were grown at 25^{o}C in the
644 dark: values shown are averages from three measurements ± standard deviation. NH_4^+-AP1
645 and NO_3^--AP1 are AP1 medium containing ammonium or nitrate, respectively, as the N-source.

Organism	Media	Growth period (days)	Copper free control	Copper wire below membrane	Copper wire above membrane
A. niger	MEA	0-8	10.82 ± 0.73	10.99 ± 0.72	10.59 ± 0.61
	NH_4^+-AP1	0-8	9.80 ± 0.84	9.70 ± 0.86	9.67 ± 0.77
	NO_3^--AP1	0-8	11.45 ± 1.10	10.85 ± 0.80	11.31 ± 0.91
B. caledonica	MEA	0-30	2.90 ± 0.17	2.33 ± 0.09	2.51 ± 0.59
	NO_3^--AP1	0-30	2.65 ± 0.11	2.67 ± 0.12	2.72 ± 0.15
	MCD	0-30	2.86 ± 0.13	2.79 ± 0.08	2.78 ± 0.12
P. javanicus	MEA	0-41	1.81 ± 0.02	1.88 ± 0.02	1.67 ± 0.02
	NO_3^--AP1	0-31	2.26 ± 0.09	2.53 ± 0.08	2.34 ± 0.09
	MCD	0-31	2.54 ± 0.06	2.43 ± 0.07	2.56 ± 0.04

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Table 2. Tolerance indices (TI) for test fungi grown on copper metal amended medium (%). Values are percentages derived from the dry biomass yield of organisms grown on media amended with copper metal by comparison with the control. All test fungi were grown at 25°C in the dark. Values shown are averages from three measurements with standard deviations.

			Tolerance index (%)	
Organism	Media	Growth period (days)	Copper wire below membrane	Copper wire on membrane
A. niger	MEA	8	109.8 ± 0.1	97.7 ± 0.1
	NH_4^+-AP1	8	106.0 ± 5.0	100.3 ± 0.7
	NO_3^--AP1	8	91.1 ± 7.0	98.2 ± 0.7
B. caledonica	MEA	30	96.6 ± 3.2	80.1 ± 5.3
	NO_3^--AP1	30	94.6 ± 0.2	92.7 ± 3.9
	MCD	30	63.9 ± 0.9	64.1 ± 0.6
P. javanicus	MEA	41	158.5 ± 22.6	151.2 ± 15
	NO_3^--AP1	31	71.2 ± 3.7	70.3 ± 5.0
	MCD	31	71.9 ± 5.4	75.9 ± 2.6

653 Table 3. Surface pH values of uninoculated agar and agar underneath fungal colonies on control
654 and copper metal amended media. *A. niger* on MEA was grown for 21 days at 25°C in the dark.
655 *A. niger* on AP1 medium was grown for 60 days at 25°C in the dark. Other fungi were grown for
656 90 days at 25°C in the dark. Test fungi were grown on unamended media as negative controls;
657 copper-amended media without test fungi were used as abiotic controls. Values shown are
658 averages ± standard deviations (n=6).

		pH values				
Organism	Media	Negative Control	Abiotic control (below) membrane	Copper below membrane	Abiotic control on membrane	Copper on membrane
A. niger	MEA	2.84 ± 0.05	5.14 ± 0.01	3.00 ± 0.10	5.19 ± 0.01	3.09 ± 0.20
	NH_4^+-AP1	1.96 ± 0.02	5.20 ± 0.01	2.90 ± 0.23	4.95 ± 0.07	2.18 ± 0.05
	NO_3^--AP1	4.57 ± 0.12	5.20 ± 0.06	5.52 ± 0.31	5.26 ± 0.06	5.22 ± 0.16
B. caledonica	MEA	8.48 ± 0.05	5.34 ± 0.01	7.73 ± 0.01	5.28 ± 0.22	7.63 ± 0.09
	NO_3^--AP1	8.89 ± 0.06	6.73 ± 0.02	8.74 ± 0.10	7.03 ± 0.03	8.87 ± 0.07
	MCD	8.75 ± 0.08	6.20 ± 0.01	8.22 ± 0.01	6.45 ± 0.07	8.26 ± 0.07
P. javanicus	MEA	7.84 ± 0.01	5.33 ± 0.07	8.39 ± 0.10	5.22 ± 0.01	8.14 ± 0.32
	NO_3^--AP1	8.95 ± 0.04	6.73 ± 0.02	8.41 ± 0.91	7.03 ± 0.03	8.80 ± 0.07
	MCD	8.57 ± 0.04	6.20 ± 0.01	8.64 ± 0.04	6.45 ± 0.07	8.34 ± 0.08

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Legends to Figures

Fig. 1. Organic acid secretion by *A. niger* in the presence or absence of copper metal in (a-c) ME or (d) NH_4^+-AP1 ammonium salt medium liquid media. Cultures were shake incubated at 125 rpm for 21 days at 25°C. (●) copper free control; (○), 10 $g \cdot L^{-1}$ copper; (□), 0.5 $g \cdot L^{-1}$ copper. Error bars are the standard deviations from at least 3 samples.

Fig. 2. Pieces of copper wire collected from ammonium salt-AP1 solid agar medium after 60 days incubation at 25 °C in the dark (a) abiotic control and (b) growth of *A. niger*. Scale bars = 50 µm. Typical images have been chosen from at least three examinations.

Fig. 3. Surface of copper sheet incubated on ammonium salt AP1 media inoculated (a, b) with or (c) without *A. niger* for 90 days at 25°C in the dark. Scale bars: a, c =10 µm, b = 100 µm. Scratches were made in the copper sheet surface using 100-grit aluminium oxide abrasive paper to enhance fungal adhesion. Typical images from several examinations are shown.

Fig. 4. SEM of copper wire pieces incubated with *A. niger* for 90 days at 25 °C in the dark (a,b) between the MEA surface and overlying cellophane membrane, or (c,d) placed on the top of the cellophane membrane. (e) abiotic control, copper metal between the membrane and agar (f) abiotic control, control copper metal on top of the the membrane. Scale bars: a, c = 50 µm, e, f = 100 µm. Fig. 4b and 4d are higher magnification images of the areas indicated by the inset squares in Fig. 4a and 4c. Scale bars: b, d = 5 µm. Typical images are shown from several examinations.

Fig. 5. Energy-dispersive X-ray analysis of copper metal wire after incubation with *A. niger* on MEA after 90 days at 25 °C in the dark. (a) copper metal placed between the cellophane membrane and agar (b) abiotic copper metal control (between membrane and agar). (c) copper metal placed ontop of the membrane. (d) abiotic copper metal control (on the membrane). Typical spectra are shown from one of at least three determinations.

Fig. 6. X-ray diffraction of minerals formed on copper metal in MEA media after incubation on MEA with *A. niger* for 90 days at 25 °C in the dark. Typical patterns are shown from one of several separate determinations.